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Preparation of recombinant ITs

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Immunotoxin-αCD40 therapy activates innate and adaptive immunity and generates a durable antitumor response in glioblastoma models

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Detailed protocol

A detailed protocol for preparing recombinant D2C7-IT can be found in published articles PMID: **23857604** and PMID: **28013405.**

D2C7-IT construction and bacterial expression

D2C7 immunotoxin (D2C7-IT) was generated by fusing the specific single-chain variable region antibody fragments (scFv) with the sequences for domains II and III of Pseudomonas exotoxin A (PE38) (pRB199-D2C7-scdsFv-PE38KDEL) (PMID: **23857604**).

The pRB199-D2C7-scdsFv-PE38KDEL plasmid was transformed into the expression host E. coli BLR (λ DE3) (Novagen-EMD Millipore, Billerica, MA), and positive clones were selected by chloramphenicol resistance and inoculated into 2.0 mL of SOC media and incubated for 2 h at 220 rpm and 37 °C.

The cells were then plated on ten LB-chloramphenicol (75 μg/mL) plates and incubated at 37 °C overnight.

The ten plates of grown bacteria were inoculated into four 2-L shake flasks, each containing 500 mL of sterile, prepared Animal Product-Free Terrific Broth media (Teknova) supplemented with 4.1 mM MgSO4, 0.5%glucose, and chloramphenicol at 34 μg/mL.

The inoculated flasks were incubated at 230 rpm and 37 °C. When the optical density at 600 nm (OD600) of bacterial culture reached an OD600 ≈ 1.8-2, D2C7-IT expression was induced with 1 mM IPTG for 2 h.

Cells were harvested by centrifugation, and the cell pellet was stored at −20 °C overnight or until lysis.

D2C7-IT inclusion body preparation

Each 500 ml bacterial cell pellet was thawed and resuspended in 100 ml of TE 50/20 buffer (50 mM Tris-Cl pH 7.5/20 mM EDTA) and dispersed through homogenization.

Next 6.5 ml of chicken egg lysozyme (Sigma) (5 mg/ml stock in ddH_2O) was added to 100 mL homogenate and incubated at room temperature for 1 h.

Twelve milliliters of 5 M NaCl (500 mM final concentration) and 9.6 mL of 25% Triton X-100 buffer (2% final concentration) were added to the bacterial lysate, which was then homogenized and incubated at room temperature for 30 min.

Finally, 4 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA) (16.7 mM final concentration) was added to the bacterial lysate, and the final volume was adjusted to 200 mL with 2% Triton-X 100 in TE 50/20 buffer (50 mM Tris–HCl pH 7.5 and 20 mM EDTA), dispersed thoroughly by homogenization, and centrifuged at 10,000 rpm at 4 °C for 50 min.

The bacterial pellets were washed (200 mL total volume) twice with 2% Triton-X 100 in TE 50/20 buffer and three times with TE 50/20 buffer. During each wash step, the bacterial pellet was thoroughly dispersed through homogenization followed by centrifugation at 10,000 rpm at 4 °C for 50 min. The final inclusion body pellets were labeled and stored at −20 °C until solubilization.

D2C7-IT inclusion body solubilization and refolding

The inclusion body pellet was dissolved in solubilization buffer (6 M guanidine–HCl) overnight at room temperature on a shaker. The solubilized inclusion body was centrifuged at 16,000 rpm at 4 °C for 50 min.

The resulting supernatant was collected, and the protein concentration was determined by Pierce Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific).

The solubilized inclusion body was then diluted to a concentration of 10 mg/mL using an appropriate volume of the solubilization buffer.

Solubilized D2C7-IT was then reduced by the addition of dithioerythritol (DTE; Sigma, St. Louis, MO) to 10 mg/mL for the final concentration and incubated for 4 h at room temperature with gentle shaking.

Reduced D2C7-IT was slowly diluted 100-fold into refolding buffer (100 mM Tris–HCl pH 8.0, 0.5 M L-arginine–HCl, 0.9 mM oxidized form of glutathione, 2 mM EDTA, 2 mL of Protease Inhibitor/L [Sigma], pH 10.3) with gentle mixing.

The refolding reaction was allowed to proceed for 72 h at 2–8 °C without agitation.

D2C7-IT purification

The refolded protein solution (3 L) was dialyzed against 50 L of dialysis buffer (100 mM Urea, 20 mM Tris–HCl, pH 7.5) overnight at 2–8 °C.

The dialyzed retentate was filtered through a 0.22-µmbottle top filter for subsequent purification.

A high-resolution (HR16/10) column (GE Healthcare) was packed with Q-Sepharose High Performance (HP) anion exchange resin (GE Healthcare) and had a packed bed volume of 20 mL.

The Q Sepharose HP column was equilibrated with ten column volumes (CVs) of buffer A (20 mM Tris–HCl, pH 7.5) until the conductivity of the effluent was within 2 mS/cm of the equilibration buffer.

The column was then run at a flow rate of 6 mL/min. The D2C7-IT Q-Sepharose HP load (3–4 L of dialyzed and filtered protein solution) was applied to the QSepharose HP column, and the flow-through was collected.

After washing the Q-Sepharose HP column with five CVs of buffer A (20 mM Tris–HCl, pH 7.5), the D2C7-IT was eluted (flow rate, 4 mL/min/fraction; total runtime, 60 min) using a linear gradient of buffer B (20 mM Tris–HCl, pH 7.5, 1 M NaCl),with tubes 1–10 collecting fractions with 0–10%buffer B, tubes 11–50 collecting fractions with 10–50% buffer B, and tubes 51–60 collecting fractions with 50–100% buffer B.

The main D2C7-IT-containing fractions eluted from QSepharose HP chromatography were pooled based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) analysis and processed for high performance liquid chromatography–size exclusion chromatography (HPLCSEC) purification.

Pooled Q-Sepharose D2C7-IT Q-Sepharose fractions were concentrated using a 30-kDa molecular weight cutoff (MWCO) Vivaspin 20 ultrafiltration device (Sartorius, Bohemia, NY).

Pooled D2C7-IT fractions were concentrated and dialyzed (using autoclaved Spectra/Por 25-kDa MWCO dialysis tubing) overnight against filter sterilized (VacuCap filters, Pall Corporation, Port Washington, NY) TSK buffer (70 mM Na2HPO4, 62.5 mM NaH2PO4, and 100 mM Na2SO4, pH 6.7).

Dialyzed D2C7-IT was then filtered through a 0.2-µm Millex GV filter (EMD Millipore) and run on a TSKgel SuperSW3000 (particle size 4 µm, 21.5 mm × 30 cm; Tosoh Bioscience, King of Prussia, PA) gel filtration column equilibrated with TSK buffer.

The column was run at a flow rate of 2 mL/min (total runtime 60 min), and when a stable base line was obtained, a 1 mL D2C7-IT sample was injected and 30-s fractions were collected under sterile conditions.

The main D2C7-IT-containing fractions eluted from the TSKgel SuperSW3000 gel filtration column were pooled based on SDS-PAGE analysis, concentrated, and filtered through a 0.2-µm Millex GV filter.

Concentrated D2C7-IT was then dialyzed overnight at 4 °C against sterile 1x PBS (Thermo Fisher Scientific),.

After dialysis D2C7-IT was filtered through a 0.2-µm Millex GV filter.

The protein concentration was assessed by Pierce Coomassie Plus (Bradford) Assay.

The final purified D2C7-IT was aliquoted and stored at −80 °C.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Chandramohan, V. (2023). Preparation of recombinant ITs. Bio-protocol Preprint. bio-protocol.org/prep2264.
2. Parker, S., McDowall, C., Sanchez-Perez, L., Osorio, C., Duncker, P. C., Briley, A., Swartz, A. M., Herndon, J. E., Yu, Y. A., McLendon, R. E., Tedder, T. F., Desjardins, A., Ashley, D. M., Gunn, M. D., Enterline, D. S., Knorr, D. A., Pastan, I. H., Nair, S. K., Bigner, D. D. and Chandramohan, V.(2023). Immunotoxin-αCD40 therapy activates innate and adaptive immunity and generates a durable antitumor response in glioblastoma models. Science Translational Medicine 15(682). DOI: 10.1126/scitranslmed.abn5649